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Citation for published version:
Kilcoyne, KR, Smith, LB, Atanassova, N, Macpherson, S, McKinnell, C, van den Driesche, S, Jobling, MS, Chambers, TJG, De Gendt, K, Verhoeven, G, O'Hara, L, Platts, S, de Franca, LR, Lara, NLM, Anderson, RA & Sharpe, RM 2014, 'Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells', *Proceedings of the National Academy of Sciences (PNAS)*, vol. 111, no. 18, pp. E1924-E1932. https://doi.org/10.1073/pnas.1320735111

Digital Object Identifier (DOI):
10.1073/pnas.1320735111

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Proceedings of the National Academy of Sciences (PNAS)

Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells

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Edited by John J. Eppig, The Jackson Laboratory, Bar Harbor, ME, and approved March 25, 2014 (received for review November 7, 2013)

Fetal growth plays a role in programming of adult cardiometabolic disorders, which in men, are associated with lowered testosterone levels. Fetal growth and fetal androgen exposure can also predetermine testosterone levels in men, although how is unknown, because the adult Leydig cells (ALCs) that produce testosterone do not differentiate until puberty. To explain this conundrum, we hypothesized that stem cells for ALCs must be present in the fetal testis and might be susceptible to programming by fetal androgen exposure during masculinization. To address this hypothesis, we used ALC ablation/regeneration to identify that, in rats, ALCs derive from stem/progenitor cells that express chicken ovalbumin upstream promoter transcription factor II. These stem cells are abundant in the fetal testis of humans and rodents, and lineage tracing in mice shows that they develop into ALCs. The stem cells also express androgen receptors (ARs). Reduction in fetal androgen action through AR KO in mice or dibutyl phthalate (DBP) -induced reduction in intratesticular testosterone in rats reduced ALC stem cell number by ∼40% at birth to adulthood and induced compensated ALC failure (low/normal testosterone and elevated luteinizing hormone). In DBP-exposed males, this failure was probably explained by reduced testicular steroidogenic acute regulatory protein expression, which is associated with increased histone methylation (H3K27me3) in the proximal promoter. Accordingly, ALCs and ALC stem cells immunoexpressed increased H3K27me3, a change that was also evident in ALC stem cells in fetal testes. These studies highlight how a key component of male reproductive development can fundamentally reprogram adult hormone production (through an epigenetic change), which might affect lifetime disease risk.

adult Leydig stem/progenitor cells | compensated Leydig cell failure | GATA4 | ethane dimethane sulfonate

Evidence that altered fetal growth/development can fundamentally alter the risk of health disorders in adulthood and perhaps, future generations continues to grow (1). Such fetal programming applies to common disorders encapsulated in the metabolic syndrome (2), which are interlinked in adult men with low testosterone levels (3). Large studies from the United States (4) and Europe (5, 6) also show that testosterone levels in men of all ages are declining with later year of birth. Aging is itself associated with declining testosterone levels and a high incidence of primary/compensated hypogonadism (7–9). Because low testosterone levels are also associated with generalized proinflammatory changes (10, 11), frailty, and risk of dying in aging men (12), what determines an adult man's testosterone level is of fundamental importance.

There is also evidence from human (13, 14) and animal experimental (15) studies that fetal programming can influence adult testosterone levels, particularly that reduced fetal androgen exposure leads to lower adult male testosterone levels (14, 15). This data fits with growing evidence that subtle deficiency in fetal androgens is a major determinant of adult male reproductive disorders, such as low sperm production (16, 17), and might explain why low sperm counts are often associated with compensated Leydig cell failure in men (18). However, the mechanisms through which fetal events could influence adult testosterone levels are unknown.

Adult Leydig cells, the main source of blood testosterone in adult males, do not develop until puberty; the only Leydig cells present in the fetal testis are a different fetal generation (19–21). Consequently, adult Leydig cells must develop from stem/progenitor cells (22, 23). Numerous studies in rodents have investigated how adult Leydig cells differentiate from progenitor cells at puberty and have identified a number of factors involved (19, 22, 24–29). However, such studies focus around the period when stem/progenitor cells have started along the differentiation pathway into adult Leydig cells. What might affect the stem/progenitor cells before this point, such as during fetal life, has not been studied, primarily because there is no agreed defining marker for such cells before they commence differentiation. In this regard, Qin et al. (30) showed that inducible KO of chicken ovalbumin upstream promoter transcription factor II (*Coup-tfII*; also termed *Nr2f2*) in prepubertal male mice resulted in failure of adult Leydig cells to develop. We, therefore, hypothesized that COUP-TFII–expressing non-Leydig interstitial cells in the testis may be the stem cells for adult Leydig cells and that effects

Significance

Men are defined by androgens (testosterone), which drive fetal masculinization (male development) and puberty and maintain masculinity in adulthood, including sex drive, erectile function, and fertility. Moreover, Western cardiometabolic diseases are all associated with lowered testosterone levels in men. Therefore, influences on testosterone levels in adulthood have pervasive importance for masculinity and health. Our study shows, for the first time, to our knowledge, that testosterone levels during fetal masculinization can (re)program adult testosterone levels through effects on stem cells, which develop into adult Leydig cells (the source of testosterone) after puberty. These stem cells are present in fetal testes of humans and animals, and using the latter, we show how these cells are reprogrammed to affect adult testosterone levels.

Author contributions: K.R.K., L.B.S., K.D.G., G.V., and R.M.S. designed research; K.R.K., N.A., S.M., C.M., S.v.d.D., M.S.J., T.J.G.C., K.D.G., L.O., S.P., L.R.d.F., N.L.M.L., and R.A.A. performed research; L.B.S., T.J.G.C., K.D.G., G.V., L.O., L.R.d.F., and R.A.A. contributed new reagents/analytic tools; K.R.K., N.A., and R.M.S. analyzed data; and K.R.K., L.B.S., G.V., R.A.A., and R.M.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1320735111/-/DCSupplemental.

on these cells might explain how fetal events can impact adult testosterone levels (adult Leydig cell function).

We presently show, through ablation/regeneration models, that adult Leydig cells in rats/mice do, indeed, derive from COUP-TFII–expressing stem cells that are numerous in the fetal testes of humans, marmosets, and rodents. We show that these stem cells express androgen receptors (ARs) and that experimental reduction of androgen production/action in fetal life in mice/rats through transgenic and chemical manipulations results in corresponding reductions in stem cell numbers in adulthood, which are accompanied by compensated adult Leydig cell failure. We also show a potential mechanism through which this programming of compensated adult Leydig cell failure might occur, namely altered histone methylation (H3K27me3) at the gene promoter for steroidogenic acute regulatory protein (*StAR*). Thus, our findings show how fetal androgen deficiency can adversely program adult Leydig cell function, which in a human context, has implications for aging, general wellbeing, and longevity of men.

Result

Identification of Adult Leydig Stem Cells by Ablation/Regeneration. Before we could investigate how fetal events might program adult Leydig cell function, we had to have a means of identifying the stem/progenitor cells from which adult Leydig cells derive. To do so, we used ethane dimethane sulfonate (EDS) -induced ablation and regeneration of Leydig cells, an established model for studying adult Leydig cell differentiation in rats (31, 32). After complete adult Leydig cell ablation by EDS (Fig. 1) and the consequent reduction in blood testosterone and elevation of blood luteinizing hormone (LH) levels (Fig. S1), there was regeneration of identifiable new adult Leydig (3β-HSD$^+$) cells from ∼14 d post-EDS, with recovery to normal adult Leydig cell numbers by 5 wk (Fig. 1). In controls, most adult Leydig (3β-HSD$^+$) cells expressed COUP-TFII in their nuclei as did regenerating adult Leydig cells (Fig. 1). Up to 1 wk post-EDS, when no identifiable adult Leydig cells were present, abundant cells with mainly spindle-shaped nuclei that expressed COUP-TFII were present. We hypothesized that adult Leydig cells regenerated from these presumptive stem cells. The number of putative stem (COUP-TFII$^+$/3β-HSD^{neg}) cells declined significantly over the 5-wk period post-EDS, consistent with some differentiating into adult Leydig cells (Fig. 1). However, there are other cell types present in or bordering the interstitium, such as macrophages, pericytes, endothelial cells, and peritubular myoid cells, which are alternative sources of regenerating adult Leydig cells, although of these cell types, only the peritubular myoid cells express COUP-TFII (33).

We, therefore, used immunohistochemistry for cell-selective markers of macrophages (CD68), pericytes (CD146), endothelial cells (CD31), and peritubular myoid cells [smooth muscle actin (SMA)] plus staining for COUP-TFII and 3β-HSD at day 6 after EDS injection. We found no evidence that regenerating adult Leydig cells coexpressed any of these cell-selective markers other than COUP-TFII (Fig. S2). We then compared the expression profile of adult Leydig cells (in controls) with the adult Leydig stem cells using a range of factors expressed in adult Leydig cells and/or reported in immature adult Leydig cells. However, of the factors investigated, only COUP-TFII and AR were shared by adult Leydig cells and their stem cells (Fig. S2).

Platelet-derived growth factor-α action through its receptor (PDGFRα) is important in adult Leydig cell differentiation from progenitors (27, 29). We found PDGFRα expression in occasional COUP-TFII$^+$ stem cells at 6 d after EDS (Fig. S3), but in control adult (Fig. S3) and fetal testes (Fig. 3), expression of PDGFRα in COUP-TFII$^+$ stem cells was rare. We examined expression of GATA4, which is essential for fetal Leydig cell development (34) and expressed in mouse (35) and human (36)

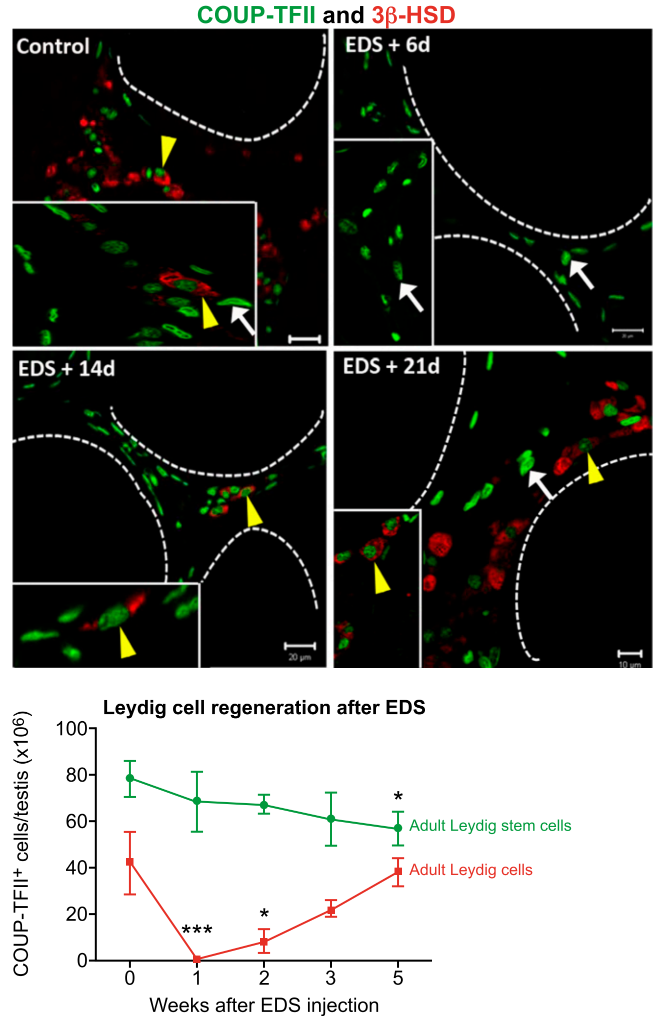

Fig. 1. Adult Leydig cell regeneration from COUP-TFII$^+$ stem cells after EDS induced Leydig cell ablation in adult rats. (*Top Left*) In controls, adult Leydig stem cells are identified by their COUP-TFII nuclear staining (green; white arrow), whereas adult Leydig cells, which also express COUP-TFII, are stained with the cytoplasmic steroidogenic enzyme marker 3β-HSD (red; yellow arrowhead). (*Top Right*) Six days after EDS injection, Leydig cells are absent, but COUP-TFII$^+$ Leydig stem cells (green nuclei) remain (white arrow). (*Middle Left*) By EDS + 14 d, some spindle-shaped COUP-TFII$^+$ Leydig stem cells (yellow arrowhead) have acquired 3β-HSD$^+$ staining (red), marking onset of adult Leydig cell regeneration. (*Middle Right*) By 21 d, numerous COUP-TFII$^+$ adult Leydig cells have regenerated. (*Bottom*, graph) Quantification of Leydig stem cells (COUP-TFII$^+$/3β-HSD^{neg}) and adult Leydig cells (COUP-TFII$^+$/3β-HSD$^+$) at various times after EDS is shown in the graph. Bars show means ± SEMs for n = 5–7 rats per group. *$P < 0.05$, ***$P < 0.001$ compared with vehicle group (time 0). Leydig stem cell numbers decline significantly by EDS + 5 wk. Images are representative of three independent experiments. *Insets* show higher magnifications of cells in transition from stem cells to adult Leydig cells. Dashed lines are the outlines of seminiferous tubules. (Scale bars: 20 μm; *Middle Right*, 10 μm.)

adult Leydig cells. At 6–14 d after EDS, a proportion of adult Leydig stem/progenitor cells (COUP-TFII$^+$), located in peritubular or other regions, coexpressed GATA4, whereas such cells (COUP-TFII$^+$/GATA4$^+$) were rarely evident in control testes (Fig. 2). At 14–21 d after EDS, these GATA4-expressing stem/progenitor cells differentiated into adult Leydig cells, which was indicated by coexpression of 3β-HSD (Fig. 2). We confirmed that this sequence mimicked normal puberty (Fig. 2). Thus, at age 10 d (before puberty), virtually all adult Leydig stem cells (COUP-TFII$^+$) were GATA4^{neg}, but at initiation of puberty (day 15), GATA4 expression

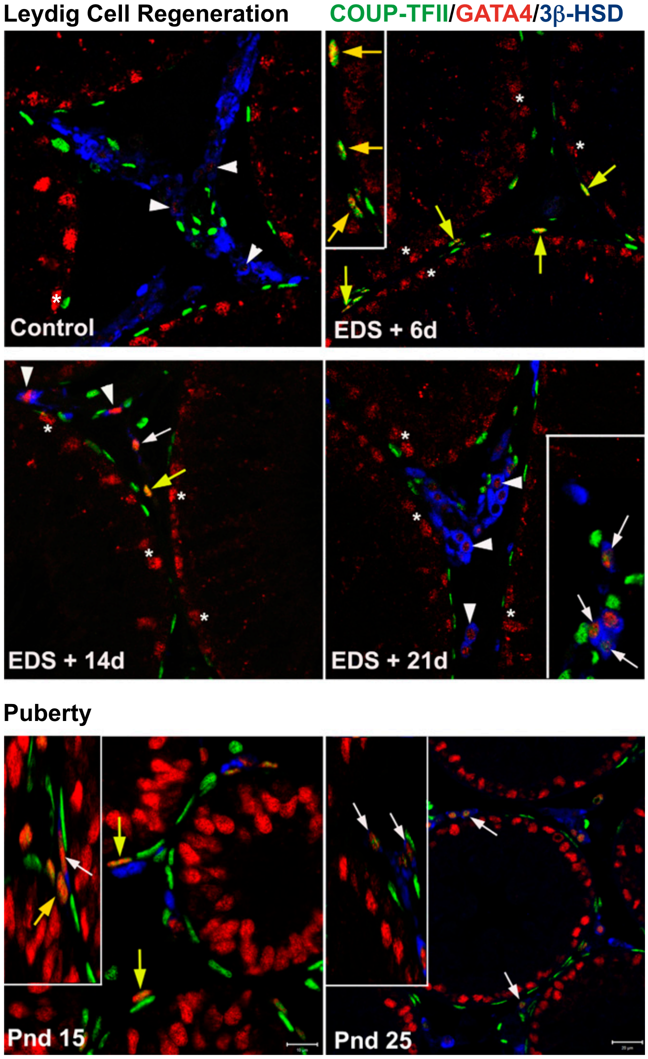

Fig. 2. Expression of GATA4 in COUP-TFII$^+$ Leydig stem cells is an early step in their differentiation into adult Leydig cells. Results are shown during Leydig cell regeneration (*Top* and *Middle*) after EDS and (*Bottom*) during normal puberty. (*Top Left*) In adult controls, GATA4 expression (red) is confined to nuclei of Sertoli cells (asterisk) and 3β-HSD$^+$ (blue) Leydig cells (white arrowheads). COUP-TFII–expressing Leydig stem cells (green) are GATA4^{neg}. (*Top Right*) Six days after EDS, Leydig cells are absent, but GATA4 has switched on in some Leydig stem cells (yellow arrows; coexpression of green and red in nuclei). (*Middle Left*) At EDS + 14 d, some of the GATA4$^+$ stem cells have switched on 3β-HSD (blue), and (*Middle Right*) by day 21, they have differentiated into adult Leydig cells. Cells coexpressing GATA4, 3β-HSD, and COUP-TFII are labeled with white arrows, but note that, for technical reasons, COUP-TFII expression in mature adult Leydig cells is often not evident in these images, whereas it is in other images that do not involve double immunostaining for GATA4 (Fig. 1). (*Bottom Left*) At the start of normal puberty (Pnd15), coexpression of GATA4 in COUP-TFII$^+$ Leydig stem cells is common (yellow arrows), and (*Bottom Right*) by Pnd25, many of these cells are also 3β-HSD$^+$ (white arrows). Images are representative of three to five animals for three independent experiments. *Insets* show higher magnifications of protein expression changes in Leydig stem cells during their differentiation into Leydig cells.

was evident in a proportion of them; by day 25, they had begun to transform into adult Leydig (3β-HSD$^+$) cells (Fig. 2). In contrast, virtually no COUP-TFII$^+$ stem cells in the fetal testis expressed GATA4, which was confined to the nuclei of Sertoli and fetal Leydig cells (Fig. 3). These studies confirmed our hypothesis that COUP-TFII–expressing non-Leydig interstitial cells must be the stem cell population from which adult Leydig cells differentiate.

Fetal Origin of Adult Leydig Stem Cells and Species Conservation. In fetal testes of humans, marmosets, rats, and mice, adult Leydig stem (COUP-TFII$^+$/3β-HSD^{neg}) cells were the most abundant cell type in the interstitium, and the majority of these cells coexpressed AR (Fig. 3). To confirm that COUP-TFII is a marker of the adult Leydig stem cell population in fetal life, we used transgenic lineage tracing. Screening of several transgenic Cre Recombinase mouse lines bred to a Cre-inducible YFP reporter gene (37) showed that the adipocyte protein 2 (aP2) Cre Recombinase (37) induced YFP expression, coincident with COUP-TFII, in adult Leydig stem cells when examined at birth (Fig. 4). Follow-up analysis showed that this fetally induced YFP expression was restricted to Leydig cells in adulthood (Fig. 4), confirming that COUP-TFII marks an interstitial stem cell population (3β-HSD^{neg}) that later develops into adult Leydig cells. Hereafter, we refer to the whole population of these cells as stem cells to avoid confusion with Leydig progenitor cells described in the literature, which we would consider as (PDGFRα$^+$/GATA4$^+$) cells just entering the Leydig cell differentiation pathway (27, 29). It is unclear if all these stem cells can develop into adult Leydig cells or only a subpopulation. However, because the adult Leydig stem cells coexpressed AR (Fig. 3), we investigated if fetal androgen deficiency might affect the development of these cells.

***Ar*KO Affects Development of Adult Leydig Stem Cells.** There is no means of specifically targeting KO of *Ar* in the adult Leydig stem cells, because the only presently known markers are COUP-TFII and AR, both of which are also expressed in peritubular myoid cells, and KO of *Ar* in the latter has phenotypic consequences, including on adult Leydig cells (38, 39). We, therefore, investigated development of adult Leydig stem cells in complete *Ar*KO mice. The results showed that, at birth and through postnatal life into adulthood, the number of adult Leydig stem cells was reduced by ∼40% in *Ar*KO males compared with WT controls (Fig. 5). There was a parallel reduction in adult Leydig cell numbers in *Ar*KO males, and they exhibited compensated Leydig cell failure based on gross distortion of their blood LH to testosterone ratio compared with controls (Fig. 5). In contrast, cell-selective KO of *Ar* in Sertoli (SC*Ar*KO) or peritubular myoid cells (PTM*Ar*KO) had no significant effect on adult Leydig stem cell numbers (Fig. S4). Although these findings suggest that androgen action on adult Leydig stem cells is important for their normal development, *Ar* is deleted in other cell types, and all testes are cryptorchid in *Ar*KOs, which are confounding factors. Therefore, we used a rat model in which intratesticular testosterone in the fetus was experimentally reduced (33) and investigated if similar effects on adult Leydig cell/stem cell development occurred, which was evident in *Ar*KOs.

Reduction in Fetal Intratesticular Testosterone Impairs Development of Adult Leydig Stem Cells. Treatment of pregnant rats from embryonic (e)13.5 to e21.5 with 500 mg/kg per day dibutyl phthalate (DBP) suppresses intratesticular testosterone (ITT) by 50–70% between e17.5 and e21.5 (33), which is confirmed presently at e21.5 (Fig. 6). This suppression was associated with a ∼40% reduction in adult Leydig stem cell number at e21.5 compared with controls, a deficit maintained through postnatal life into adulthood (Fig. 6). In contrast to *Ar*KOs, DBP treatment did not alter final adult Leydig cell number (Fig. 6). Despite normal Leydig cell numbers and significantly raised blood LH levels in DBP-exposed males in adulthood, blood testosterone levels were significantly reduced, resulting in a distorted LH to testosterone ratio compared with controls, indicative of compensated Leydig cell failure (Fig. 6). These results suggest that fetal ITT affects adult Leydig stem cell number and their functional competence when they differentiate into adult Leydig cells. Regarding the former, the number of adult Leydig stem (COUP-TFII$^+$/3β-HSD^{neg}) cells increased 17-fold from e17.5 [$0.16 \pm 0.03 \times 10^6$ (mean ± SEM),

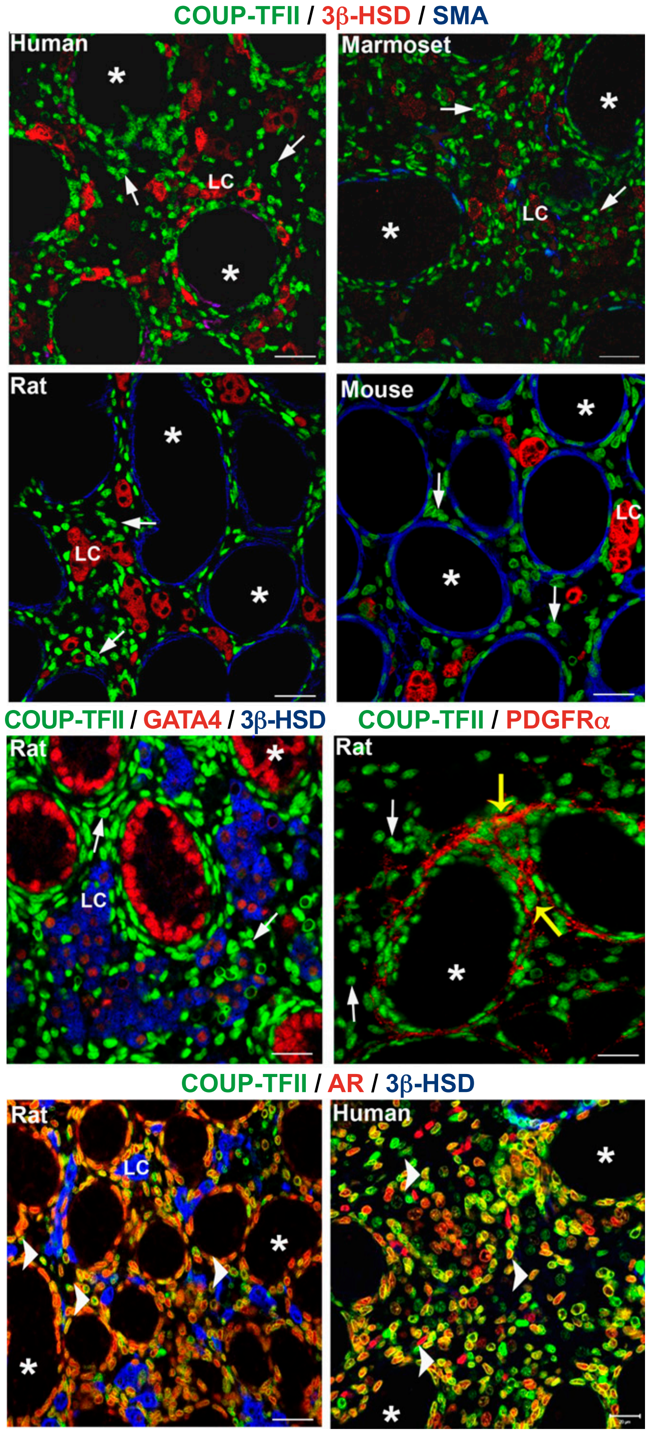

Fig. 3. Presence of adult Leydig stem cells (white arrows) expressing COUP-TFII (green) in their nuclei in fetal testes of different species (rows 1 and 2) and their protein expression phenotype (rows 3 and 4). In rows 1 and 2, red indicates 3β-HSD [fetal Leydig cells (LCs)], and blue indicates SMA (asterisks are seminiferous cords). Rows 3 and 4 show that, whereas most adult Leydig stem (COUP-TFII$^+$) cells lack expression of PDGFRα and GATA4 (white arrows), most adult Leydig stem cells coexpress (white arrowheads) the AR (red). In rows 3 and 4, blue indicates 3β-HSD (fetal LC). Yellow arrows show potential PDGFRα$^+$ adult Leydig stem cells. Images are representative of three to five animals from three independent experiments. (Scale bars: 20 μm.)

n = 4] to e21.5 (2.81 ± 0.24 × 10^6, n = 7) in control rats (this study) when ITT is high/increasing (33, 40).

Potential Mechanism for Fetal Programming of Compensated Adult Leydig Cell Failure. We used the rat DBP model to investigate expression of six genes in the steroidogenic pathway in the adult testis (Fig. 7). Because we used scrotal and cryptorchid testes from DBP-exposed animals for these studies, we used a control gene (*Sox9*) expressed specifically in Sertoli cells to correct expression of the target genes, because Leydig cell mRNAs would be overrepresented in cryptorchid vs. scrotal testes because of massive germ cell loss in the former. *Sox9* was chosen, because adult Sertoli cell number is unchanged in DBP-exposed animals, irrespective of whether testes are scrotal or cryptorchid (41). In DBP-exposed animals, expression of *Lhcgr*, *Cyp11a1*, *Cyp17a1*, and *17β-hsd3* were unchanged, whereas expression of *StAR* and *3β-hsd* were both significantly reduced compared with controls (Fig. 7). Because StAR is one of the factors involved in cholesterol transport into the mitochondrion (42), which is rate-limiting for steroidogenesis (43, 44), this change was considered the most significant.

For fetal androgen action on stem cells to alter subsequent adult Leydig cell function through repression of *StAR* transcription, we considered an epigenetic mechanism likely. Altered methylation of the proximal-1 promoter region of *StAR* is crucial for regulating its expression (45–47) and conserved across species (48). Because H3K27me3 is an established transcriptional repressor (49–51), including of *StAR* (47), we investigated if the level of H3K27me3 upstream of the coding region of *StAR* was altered using a ChIP assay (47, 51).

Our ChIP results showed a significant increase in H3K27me3 localization to the *StAR* proximal promoter in adult testes of DBP-exposed animals compared with controls (Fig. 8). This increase in repressive H3K27me3 could account for reduced *StAR* expression. Using an antibody against H3K27me3, we showed that a proportion of Leydig cells in DBP-exposed rats at postnatal day 25 (Pnd25) and in adulthood showed expression of H3K27me3 in their nuclei, whereas it was minimal/absent in controls at this antibody dilution (Fig. 9); a similar difference was

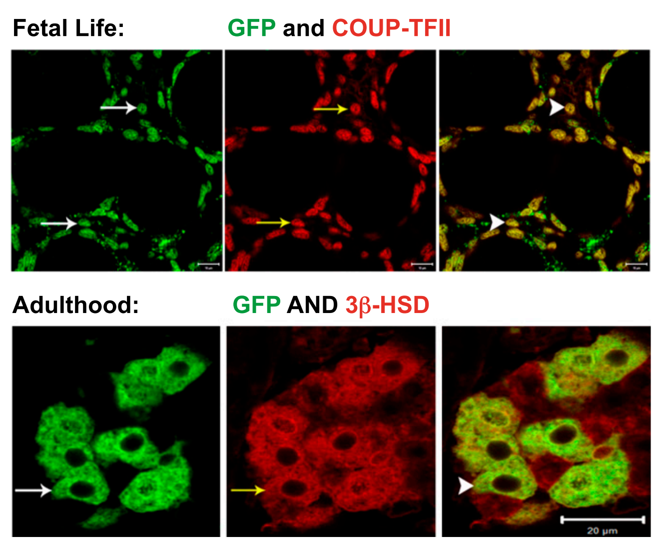

Fig. 4. COUP-TFII is a marker of the adult Leydig stem population, which is evident from the transgenic lineage tracing of adult Leydig cells. (*Upper*) At birth, the aP2 Cre Recombinase-induced GFP expression (white arrows) is coincident with COUP-TFII expression (yellow arrows). The merged image shows COUP-TFII and GFP colocalization (white arrowheads) in adult Leydig stem cells. (*Lower*) In adulthood, fetally induced YFP expression (white arrow) is restricted to 3β-HSD–expressing (yellow arrow) adult Leydig cells, which is indicated by their coexpression (white arrowhead in merged image). Images are representative of three to five animals from two independent experiments. (Scale bars: 20 μm.)

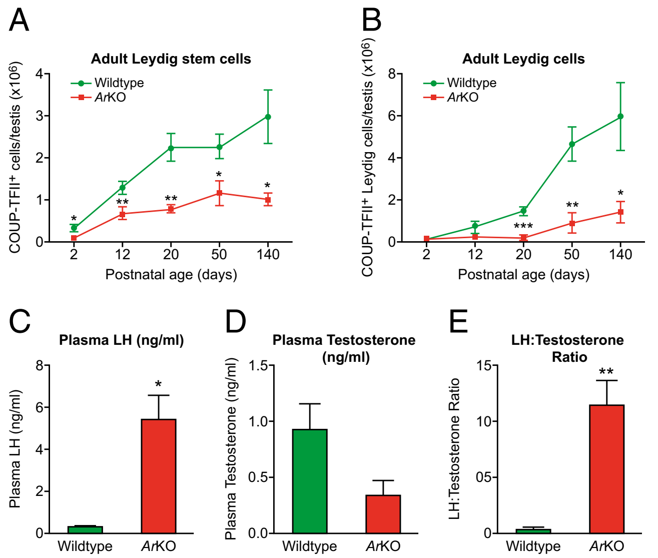

Fig. 5. Effect of complete *Ar*KO in mice on numerical development and function of adult Leydig cells and their stem cells. (*A*) Adult Leydig stem cells (COUP-TFII^{+}/3β-HSD^{neg}) and (*B*) adult Leydig cells (COUP-TFII^{+}/3β-HSD^{+}) in *Ar*KO mice were quantified. Plasma hormone levels in adulthood as a measure of adult Leydig cell function are shown for (*C*) LH, (*D*) testosterone, and (*E*) the LH to testosterone ratio. Values are means ± SEMs for n = 7–10 WT and *Ar*KO mice at each age. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with respective (control) WT value.

found between *Ar*KO and WT adult mice (Fig. 9). This increased H3K27me3 immunoexpression may not be restricted to *StAR*, but consistent with the hypothesized fetal origin of the change in H3K27me3 localization to the *StAR* promoter in testes of DBP-exposed animals, we found localization of H3K27me3 to adult Leydig stem (COUP-TFII^{+}) cells in the fetal testes of DBP-exposed rats, whereas no/minimal expression was detectable in stem cells in controls at this antibody dilution (Fig. 9). In contrast, immunoexpression of unmodified histone 3 was comparable in control and DBP-exposed animals (Fig. S5). These observations provide a potential mechanism (i.e., H3K27me3) through which deficiency in fetal androgen action on stem cells could reprogram/compromise adult Leydig cell function by altering transcription of *StAR*.

Discussion

Fetal programming of testosterone levels/Leydig cell function in adult men is conceptually important because of its close interrelationship with common diseases that impair quality and length of life in men. Although there is indirect supporting evidence for fetal programming of adult testosterone levels (reviewed in ref. 16), a limiting factor is the absence of direct evidence for a mechanism to explain how adult Leydig cells, which do not appear until puberty, could be affected (programmed) by fetal events. The present findings provide this evidence in animal studies by (*i*) identifying a population of (COUP-TFII–expressing) stem cells from which adult Leydig cells differentiate, (*ii*) showing that these cells are numerous in the fetal testis and conserved across species, (*iii*) showing that these cells are androgen targets, and (*iv*) showing that fetal deficits in intratesticular levels/action impair development of these stem cells, resulting in impaired function of the adult Leydig cells that differentiate from them. An epigenetic mechanism that might explain this long-range programming through altered H3K27me3 of the *StAR* promoter is also shown.

Our initial goal, inspired by the KO studies by Qin et al. (30), was to identify if COUP-TFII–expressing non-Leydig interstitial cells were stem cells for adult Leydig cells. Using EDS-induced adult Leydig cell ablation, we show that the new generation of adult Leydig cells differentiates from among the population of COUP-TFII–expressing, undifferentiated, spindle-shaped interstitial cells. These cells, which we have termed adult Leydig stem cells, do not express classical Leydig cell markers (LH receptor, steroidogenic factor-1, steroidogenic enzymes, and INSl3) but express COUP-TFII and AR, which they share with adult Leydig cells (although neither of these markers is Leydig cell-specific). Based on comparative phenotyping of the stem cells and newly differentiating Leydig cells after EDS, switching on of the transcription factor GATA4 and probably, PDGFRα seems to be a key early differentiation step, and thereafter, these GATA4^{+}/COUP-TFII^{+} cells switch on classic Leydig cell markers, such as 3β-HSD and INSl3. We show that this differentiation pattern recapitulates what happens during normal puberty in the rat. GATA4 is important for differentiation of fetal Leydig cells (34), which also derive from COUP-TFII–expressing interstitial cells (33). GATA4 may also be imperative for development of adult Leydig cells (52), because it induces expression of steroidogenic factor-1 and StAR (53). Our findings are consistent with previous reports of GATA4 expression in adult Leydig cells in humans and rats (27, 35, 36) as well as in adult Leydig stem cells (27). Expression of PDGFRα was not explored in detail in our studies, but our findings and previous studies (27, 29) point to a similar pattern and timing of expression in the adult Leydig stem cells, which was detailed for GATA4. Our interpretation is that

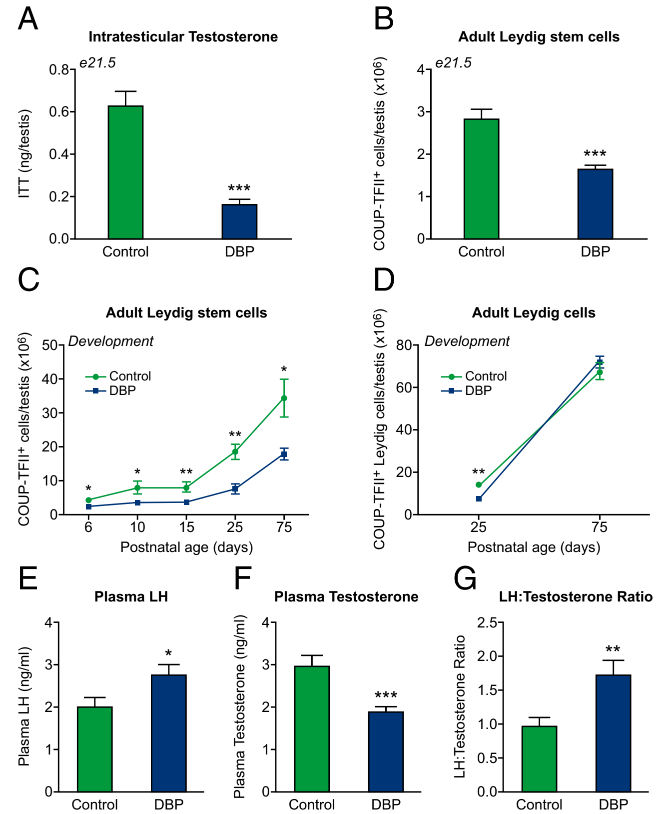

Fig. 6. DBP-induced reduction in fetal ITT alters numerical development of adult Leydig stem cells and results in compensated Leydig cell failure in adulthood. (*A*) ITT levels and (*B*) adult Leydig stem cells (COUP-TFII^{+}/3β-HSD^{neg}) at e21.5 in relation to numerical development of (*C*) adult Leydig stem cells and (*D*) adult Leydig cells (COUP-TFII^{+}/3β-HSD^{+}) postnatally through to adulthood. Plasma hormone levels in adulthood as a measure of adult Leydig cell function are shown for (*E*) LH, (*F*) testosterone, and (*G*) the LH to testosterone ratio. Values are means ± SEMs for n = 6–8 rats in each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with respective (control) vehicle value.

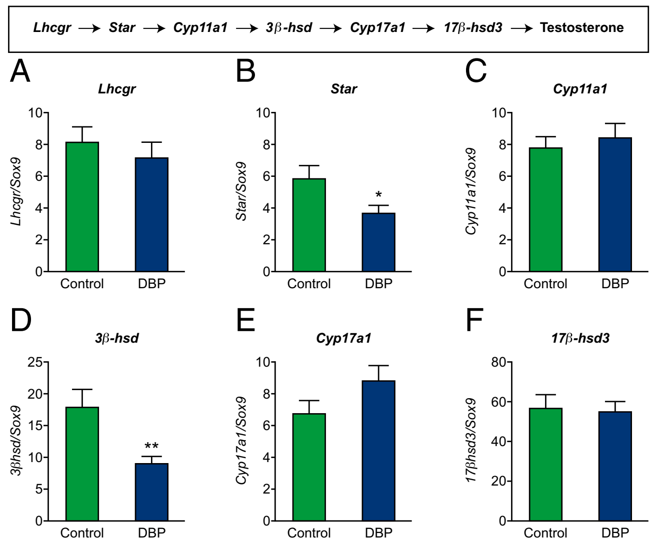

Fig. 7. Effect of prenatal DBP exposure on adult Leydig cell function as monitored by expression of Leydig cell-specific genes in the steroidogenic pathway. The testosterone synthesis cascade is shown at the top starting with the LH receptor (*Lhcgr*) and finishing with 17β-hydroxysteroid dehydrogenase type 3 (*17β-hsd3*). (*A*) *LHcgr*, (*B*) *Star*, (*C*) *Cyp11a1*, (*D*) *3β-hsd*, (*E*) *Cyp17a1*, (*F*) *17β-hsd3*. Because testis cellular composition was affected by treatment (loss of germ cells and their mRNA in cryptorchid testes) in some DBP-exposed animals, gene expression has been expressed relative to *Sox9* expression in the same testis (in the text). Values are means ± SEMs for n = 11–12 rats in each group. *P < 0.05, **P < 0.01 compared with respective (control) vehicle.

PDGFRα and GATA4 are switched on in the stem cells only when they commence differentiation down the adult Leydig cell pathway (i.e., become progenitors), because few, if any, of the (COUP-TFII$^+$) stem cells in the fetal testis express either GATA4 or PDGFRα. It is unclear if the complete population of COUP-TFII–expressing stem cells can develop into adult Leydig cells or only a subpopulation, because only a proportion of the stem cells switched on GATA4 or PDGFRα after EDS.

Previous studies suggested that adult Leydig cells could originate from other interstitial cell types, such as peritubular myoid cells, pericytes, endothelial cells, or macrophages (24, 29, 54–56). Using specific markers for these cell types and the EDS model, we found no evidence for coexpression of COUP-TFII in these cell types. Therefore, because the majority of normal and regenerating adult Leydig cells expresses COUP-TFII, we concluded that development of adult Leydig cells from these other cell types, although possible, is probably not the main mechanism. This conclusion is supported by our lineage tracing experiment. In keeping with earlier studies (27, 29, 57), we noted that adult Leydig cells commonly derive from (COUP-TFII$^+$) cells that border the seminiferous tubules, although in contrast to other studies (29), we did not find any cells coexpressing the peritubular myoid (PTM) cell marker SMA, COUP-TFII, and early Leydig cell markers. However, our findings do not exclude the possibility that, after EDS treatment, regenerating adult Leydig cells could also derive from interstitial cells that have dedifferentiated (e.g., pericytes that have switched off CD146 or PTM cells that have switched off SMA) and then either switched on COUP-TFII (pericytes) or maintained their COUP-TFII expression (PTM cells).

Our results show an abundance of adult Leydig stem cells in the fetal testes of four species, including humans, and that most of these cells coexpress AR; similar cells are present in the adult testes of these species together with COUP-TFII–expressing adult Leydig cells. Our data show that, in fetal life, these stem cells increase >17-fold in number from e15.5 to e21.5 in rats when intratesticular testosterone levels are high/increasing (33, 40). Moreover, KO of *Ar* in this cell type (and in all other cells; *Ar*KOs) in mice or experimental lowering of intratesticular testosterone in rats throughout this period (DBP exposure) both resulted in ∼40% reduction in numbers of adult Leydig stem cells around birth. This finding suggests that androgens positively regulate proliferation of the stem cells. In both of our experimental situations involving deficient fetal androgen action, the resulting decrease in stem cell number persisted through to adulthood, despite the fact that puberty was associated with a marked and parallel increase in stem cell number in control and treated rats and mice. This observation indicates that factors other than androgens play an important role during puberty in determining adult Leydig stem cell proliferation/number. The increase in COUP-TFII–expressing stem cells during puberty is consistent with an earlier study that quantified mesenchymal non-Leydig interstitial cells based on morphology (22).

We anticipated that a developmental deficit in adult Leydig stem cells might lead to reduced adult Leydig cell number. Although such a reduction was the case in *Ar*KOs, normal numbers of adult Leydig cells developed in DBP-exposed rats in which androgen levels/action had been reduced just in fetal life. One explanation for this difference in outcome, despite the similarity in shortfall of adult Leydig stem cells, is that KO of *Ar* in other testis cell types in *Ar*KO mice caused additional effects on adult Leydig cell differentiation. Indeed, because SC*Ar*KO mice (deficient in SC*Ar*) also exhibit a numerical deficit in adult Leydig cells as well as *Ar*KOs (58), it is a potential explanation. Nevertheless, the absence of any significant effect on adult Leydig stem cell numbers in SC*Ar*KO or PTM*Ar*KO (deficient in PTM*Ar*) mice [but their reduction in *Ar*KOs (which lack AR also in the adult Leydig stem cells) and DBP-exposed rats] emphasizes the importance of androgen action on the stem cells. In this regard, we cannot exclude the possibility that the fetal reduction in adult Leydig stem cells is secondary to reduced Sertoli cell number (as a result of androgen deficiency); as such, a reduction is evident in both *Ar*KO mice and DBP-exposed rats at birth (59).

In both models in which there was a fetal deficit in androgen levels/action, there was compensated adult Leydig cell failure

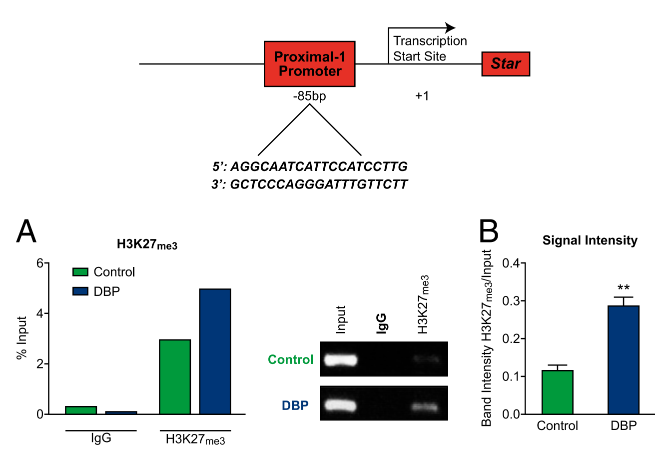

Fig. 8. Effect of prenatal DBP exposure on H3K27me3 in the proximal promoter of *StAR*. The schematic shows the *StAR* promoter region targeted (−85 bp) and amplified by PCR using primers as outlined (167 bp in length). Levels of H3K27me3 were increased at the proximal promoter of *StAR*, which was analyzed by ChIP (*A*) and densitometric assessment of PCR products (*B*). Normal rabbit IgG was used as the negative control. Data were calculated using the percentage total genomic input method. The input DNA threshold cycle value (i.e., DNA that did not undergo immunoprecipitation) was used to normalize the ChIP data. The average threshold cycle for triplicate assays was used in all subsequent calculations. Values are means ± SEMs for n = 3 per group. **P < 0.01 compared with (control) vehicle.

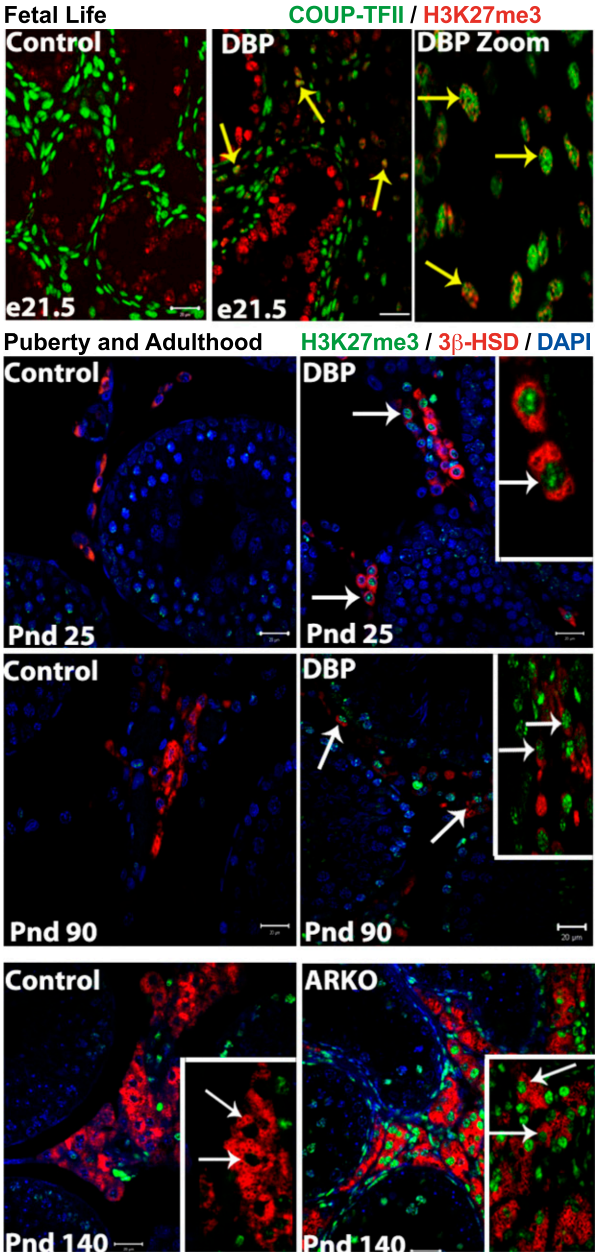

Fig. 9. Effect of fetal DBP exposure or *Ar*KO on H3K27me3 expression in adult Leydig cells and their stem cells in the rat/mouse testis. (Row 1) A proportion of adult Leydig stem cells (yellow arrows) coexpresses COUP-TFII (green) and H3K27me3 (red) in their nuclei in DBP-exposed rats at e21.5 (*Center* and *Right*), whereas in controls, H3K27me3 was detected only in Sertoli cells at this antibody concentration (*Left*). (Rows 2–4) During puberty (Pnd25) and adulthood, adult Leydig cells (3β-HSD$^+$; red cytoplasmic staining) in DBP-exposed rats or *Ar*KO mice expressed higher levels of H3K27me3 in their nuclei (green; white arrows) compared with controls, in which it was minimal/absent (*Left*). Images are representative of three to five animals for each group for three independent experiments. (Scale bars: 20 μm.)

defined as normal/reduced blood testosterone in the face of elevated LH and thus, an altered LH to testosterone ratio (7, 60). Because adult Leydig cells develop from the stem cells, this finding implies that the stem cells are modified functionally as well as numerically because of reduced fetal androgen exposure, and this ultimately translates in adulthood into compromised steroidogenesis. Based on results from the DBP rat model, a potential explanation is reduced expression of *StAR*, which contributes to regulating the import of cholesterol into the mitochondrion, the rate-limiting step for testosterone production (42–44). Expression of other genes involved in Leydig cell steroidogenesis was unaffected by in utero DBP exposure apart from *3β-hsd*, which was also decreased, but this change might not be expected to significantly impact steroidogenesis, because it is not considered rate-limiting.

To investigate a potential mechanism for reduced expression of *StAR*, we hypothesized that it was because of histone modifications in its proximal-1 promoter region, which are important in its regulation (45–47). Using ChIP, we showed that H3K27me3 upstream of the coding region of *StAR*, which is associated with transcriptional repression (47, 49–51), was increased in testes of DBP-exposed rats. Consistent with this finding, we found increased immunoexpression of H3K27me3 in adult Leydig cells of DBP-exposed rats and their stem cells in fetal life. A similar increase in H3K27me3 immunoexpression in adult Leydig cells of *Ar*KOs is consistent with it being because of (fetal) androgen deficiency rather than DBP exposure. Altered H3K27me3 of the *StAR* proximal promoter region in stem cells in fetal life potentially explains the compensated Leydig cell failure of DBP-exposed rats in adulthood. In this regard, two other facts are important. First, H3K27me3 is propagated through cell divisions (61, 62); therefore, it is reasonable to suppose that H3K27me3-induced modification of the *StAR* promoter in adult Leydig stem cells in fetal life would be transmitted through to adulthood, despite the huge proliferative changes that occur in stem cells pre- and postnatally. Second, LH is a positive regulator of *StAR* (47, 63, 64), and therefore, our finding that *StAR* expression is reduced in adulthood in testes of DBP-exposed rats, despite elevated blood LH levels (and normal *Lhr* expression), is additional indirect evidence that altered responsiveness of *StAR* has occurred. However, our studies do not rule out that other epigenetic factors might be altered that could also lead to altered expression of *StAR* or *3β-hsd*.

Alternative explanations for our observations are possible. For example, fetal phthalate exposure of rats has been shown to alter adrenal function in adulthood (65), which may secondarily alter Leydig cell function (66). We cannot exclude this possibility as a contributory factor in our DBP studies, although it would not explain the similar changes observed in *Ar*KO mice.

Our finding that fetal deficits in androgen action can result in compromised adult Leydig cell function in rodents fits with emerging evidence from humans (14, 16). There is a similar connection between reduced fetal androgens and reduced adult sperm counts/sperm production in men and rats (15, 16). What further ties these observations together is that men with low sperm counts commonly exhibit compensated adult Leydig cell failure, although why is unknown (18, 60, 67). It has been suggested that it may be indicative of a common underlying (fetal) cause (18), and our present animal experimental studies provide direct supporting evidence for this suggestion. This suggested connection has widespread implications, because one in six young men in northern European countries has a low sperm count (<20 million/mL) (68), and testosterone levels in men of all ages are declining (4–6). Moreover, it has implications for morbidities associated with aging and the aging-related decline in testosterone levels (3, 7–9, 69), because ∼10% of aging men exhibit compensated Leydig cell failure (7). Our findings also provide a pathway through which fetal growth/birth weight could influence testosterone levels in adult men (13), because no explanation for this association is currently available. Finally, our findings add a new dimension to the substantial body of evidence (16) by showing how deficits in fetal androgen exposure lead to a range of adverse changes in reproductive function and disorders in boys/men.

Materials and Methods

Animals and Treatments. Selective destruction of Leydig cells in adult Wistar rats used a single i.p. injection of EDS [75 mg EDS/kg in 2 mL/kg DMSO:water (1:3; vol:

vol)] (31, 32); control rats received vehicle. Rats were maintained under standard controlled conditions according to United Kingdom Home Office Guidelines (including an ethical approval step) and fed a soy-free breeding diet [RM3 (E) soya free; SDS]. Groups of five to seven vehicle- and EDS-treated rats were killed at 6, 14, 21, and 35 d after injection. To investigate if reduced fetal testosterone levels affect development of adult Leydig stem cells, time-mated pregnant rats were administered DBP (Sigma-Aldrich) at 500 mg/kg per day by oral gavage in 1 mL/kg corn oil from e13.5 to e21.5. This treatment reduces fetal intratesticular testosterone by 40–80% (33). Groups of 6–12 vehicle- and DBP-exposed rats were killed at e21.5 and Pnd6, -10, -15, -25, and -75.

Human, Marmoset, and Mouse Fetal Testis Tissue. Second trimester (14–18 wk) human fetal testes were obtained after termination of pregnancy. Women gave consent in accordance with United Kingdom national guidelines (70), and ethical approval was obtained from the local research ethics committee (71). Marmoset fetal testes (72) and mouse fetal testes (e18.5) (33) were obtained as described earlier.

Lineage Tracing of Adult Leydig Stem Cells. Male congenic C57BL/6J mice hemizygous for an *aP2*-Cre transgene (37) were mated to homozygous R26R-EYFP females (73). The +/*aP2*-Cre:+/R26R-EYFP and $^{+/+}$:+/R26R-EYFP (control) male offspring from these matings were genotyped as previously described (38).

Generation of *Ar*KO Mice. *Ar*KO mice were generated (58) by crossing *Ar*flox/+ females with phosphoglycerate kinase-1–Cre$^{+/+}$ males. Animals were treated according to the Care and Use of Laboratory Animals of the Catholic University of Leuven with approval by the local ethics committee. Groups of 5–11 WT and *Ar*KO males were killed on Pnd2, -12, -20, -50, and -140.

Tissue Collection and Processing. Animals were killed by CO_2 inhalation and cervical dislocation. In adults, blood was collected by cardiac puncture into a heparinized syringe. Testes were dissected, weighed, and either frozen for RNA analysis or fixed in Bouins (6 h) before processing into paraffin wax (33). Sections (5 μm) were mounted on charged microscope slides (VWR) and dried overnight at 50 °C.

Immunohistochemical Analysis. Immunostaining was for two to three proteins to delineate cell types for analysis using methods and antibodies validated previously (33, 41). Slides were dewaxed and rehydrated and underwent heat-induced antigen retrieval (33). Sections were blocked using 20% normal rabbit serum (vol/vol; Biosera) and 5% BSA (wt/vol; Sigma-Aldrich) in Tris-buffered saline (TBS). Secondary antibodies were diluted 1:500 in sera (Table S1). Slides were incubated for 30 min with Streptavidin-alkaline phosphatase (Vector) at 1:200 in TBS. Fast blue and red were used for protein detection (Perma Blue/Red; Diagnostic BioSystems) followed by antigen retrieval for 2.5 min on medium heat. Sections were reblocked in serum/TBS/BSA as above before application of (second) primary antibody followed by repetition of the above process. Streptavidin-HRP (Dako) at 1:1,000 in TBS was used for detection of biotinylated secondaries (1:500) in TBS. Detection used 3,3-diaminobenzidine tetrahydrochloride (Dako). Slides were mounted with Permafluor (Thermo Scientific). Each immunohistochemistry run included negative controls, with replacement of the primary antibody by blocking serum or preabsorption (PDGFRα) by incubation at 4 °C overnight with 10× immunogen concentration (Santa Cruz) (Fig. S6). Sections from control and treatment groups were mounted on the same slide where possible; each experiment used sections from three to six animals per group/age. For immunofluorescence, primary and secondary antibodies were diluted as optimized (Table S1). Detection used tyramide (Tyr-Cy3/5; Perkin-Elmer-TSA-Plus Cyanine3/5 System; Perkin-Elmer Life Sciences) for 10 min (1:50) in its buffer. Nuclear counterstain (DAPI; Sigma-Aldrich) was diluted 1:500 in TBS and incubated for 10 min. For colocalization, normal chicken serum was used as blocking serum to prevent cross-reaction with mouse/rabbit/goat antibodies, and the protocol was continued as before. Fluorescent images were captured using the laser scanning confocal microscope 710 Axiovert Observer Z1 (Carl Zeiss). Images were compiled using Photoshop 7.0 (Adobe Systems Inc.).

Identification of Testis Cell Types. Leydig cells were identified as cells immunopositive for 3β-HSD. SMA distinguished peritubular myoid cells and defined seminiferous cords/tubules. Macrophages, pericytes, and endothelial cells were identified using specific markers (Table S1). COUP-TFII^{+} interstitial cells immunonegative for the aforementioned cell-specific markers were considered adult Leydig stem cells.

Quantification of Adult Leydig Cells and Adult Leydig Stem Cells. Numbers per testis of COUP-TFII^{+} adult Leydig stem cells and adult Leydig cells were determined by stereology (33, 41). A Zeiss Axio-Imager microscope (Carl Zeiss) fitted with a Hitachi HVC20 camera (Hitachi Denshi Europe) and a Prior automatic stage (Prior Scientific Instruments Ltd.) was used plus Image-Pro Plus v7.0 with Stereologer Analyzer Pro (Media Cybernetics). Using random fields, testis cross-sections were analyzed for COUP-TFII^{+}/3β-HSD^{neg} adult Leydig stem cells and adult Leydig cells (3β-HSD^{+}) and expressed as relative volumes per testis before conversion to absolute volumes using testis weight; then, they were converted to cell number per testis using the average nuclear diameter (~150 nuclei) measured for each sample, group, and age (33, 41).

RNA Extraction. Total RNA was isolated from frozen testes using the RNeasy Mini Extraction Kit with RNase-Free DNase (Qiagen) as per the manufacturer's instructions. RNA quality and concentration were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Random hexamer primed cDNA was prepared using SuperScript VILO cDNA (Invitrogen) as per the manufacturer's instructions and stored at −20 °C.

Quantitative Gene Expression Analysis. Quantitative PCR was performed for genes (Table S2) using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) and optimized standard conditions and probes (Roche Universal Probe Library), and it was expressed relative to internal 18s control (quantified using the ΔΔthreshold cycle method). The mean of triplicates per sample was determined and standardized relative to adult control testis (Ambion).

Hormone Analysis. Plasma LH and testosterone were measured using assays detailed previously (33, 74). All samples from each experiment were run in a single assay, and the within-assay coefficients of variation were <10%.

ChIP Assay. Testis tissue (150 mg) from adult control and DBP-exposed rats was suspended in PBS and incubated with 37% (vol/vol) formaldehyde (270 μL) for 10 min before quenching with glycine (250 μL 2.5 M; Sigma-Aldrich). After centrifugation, the pellet was resuspended in PBS and protease inhibitor mixture (Roche Complete) and homogenized (T10 Basic Ultra-Turrax; IKA). Samples were sonicated using a Bioruptor (Diagenode) to shear chromatin to 200–1,000 bp before diluting 10-fold in immunoprecipitation dilution buffer [0.1% SDS, 1.1% Triton-X (wt/vol), 1.2 mM EDTA, 16.7 mM Tris·HCl, 167 mM NaCl]. An aliquot (1%) was saved for input control. Samples were precleaned using salmon sperm DNA/Protein Agarose A (Millipore) for 30 min at 4 °C with rotation and then incubated overnight (with rotation at 4 °C) with H3K27me3 antibody, rabbit IgG (27472; Abcam) as negative control, or anti-histone 3 (Table S1) as positive control. Samples were incubated with salmon sperm DNA/Protein Agarose A for 4 h at 4 °C. After centrifugation, precipitates were washed for 10 min with solutions of low salt (0.1% SDS, 1% Triton-X, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), high salt (0.1% SDS, 1% Triton-X, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), lithium chloride (1 mM EDTA, 10 mM Tris·HCl, 1% Deoxycholic acid, 1% Igepal ca630, 0.25 M LiCl), and Tris-ethylendiaminetetraacetic acid buffer (1 mM EDTA, 10 mM Tris·HCl). Chromatin was eluted in 1% SDS and 0.1 M $NaHCO_3$. NaCl (8 μL 5 M) was added to the eluate and incubated overnight at 4 °C to reverse cross-links before incubating with 0.5 M EDTA, 1 M Tris·HCL, and proteinase K for 60 min at 45 °C. Purified DNA from samples, including input control, was recovered (QIAquick PCR Purification Kit; Qiagen). Primers for the proximal upstream promoter of *StAR* (167 bp) (Table S2) were used for PCR using SYBR Green Master Mix (Brilliant III Ultrafast; Agilent Technologies). PCR products were visualized by 2% agarose gel electrophoresis followed by densitometric analysis using ImageJ (version 1.46h; National Institutes of Health).

Statistical Analysis. Data were analyzed by GraphPad Prism (version 5; GraphPad Software, Inc.) using ANOVA followed by either posthoc Bonferroni or Student *t* tests as appropriate. Where significant heterogeneity of variance occurred (e.g., plasma testosterone), values were log-transformed before analysis.

ACKNOWLEDGMENTS. We thank Professor Ian Morris for the gift of ethane dimethane sulfonate and William Mungall for assistance with the animal work. This work was supported by United Kingdom Medical Research Council Grants G1100354/1 (to L.B.S.), G1100357 (to R.A.A.), and G33253 (to R.M.S.).

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